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# Assessment of Pandemic and Seasonal Influenza A (H1N1) Virus Susceptibility to Neuraminidase Inhibitors in Three Enzyme Activity Inhibition Assays▽†

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**The neuraminidase inhibitors (NAIs) zanamivir and oseltamivir are currently the only antiviral drugs effective for the treatment and prophylaxis of 2009 pandemic influenza A (H1N1) virus infections. The proven potential of these viruses to acquire NAI resistance during treatment emphasizes the need to assess their NAI susceptibility. The 50% inhibitory concentrations ($IC_{50}$s) are known to vary depending on the neuraminidase inhibition (NI) test used; however, few side-by-side comparisons of different NI assays have been done. In the present study, a panel of 11 isolates representing 2009 seasonal and pandemic influenza H1N1 viruses, including oseltamivir-resistant H275Y variants, were tested in three functional NI assays: chemiluminescent (CL), fluorescent (FL), and colorimetric (CM). The sensitivities of the viruses to zanamivir, oseltamivir, and three investigational NAIs (peramivir, R-125489, and A-315675) were assessed. All isolates with the exception of H275Y variants were sensitive to all five NAIs by all three NI assays. The H275Y variants showed substantially elevated $IC_{50}$s against oseltamivir and peramivir. The three NI assays generally yielded consistent results; thus, the choice of NI assay does not appear to affect conclusions based on drug susceptibility surveillance. Each assay, however, offers certain advantages compared to the others: the CL assay required less virus volume and the FL assay provided the greatest difference in the $IC_{50}$s between the wild type and the variants, whereas the $IC_{50}$s obtained from the CM assay may be the most predictive of the drug concentrations needed to inhibit enzyme activity in humans. It would be desirable to develop an NI assay which combines the advantages of all three currently available assays but which lacks their shortcomings.**

For the treatment and chemoprophylaxis of infections caused by influenza A viruses, the U.S. Food and Drug Administration (FDA) has approved four drugs: amantadine and rimantadine as well as zanamivir and oseltamivir. These drugs belong to two classes, adamantanes (i.e., M2 ion-channel blockers) and neuraminidase (NA) inhibitors (NAIs), respectively. In recent years, the effectiveness of M2 blockers has been greatly compromised, which limits their usefulness in clinical practice. This is largely due to the rapid emergence and widespread circulation of adamantane-resistant influenza viruses (1, 5, 6, 7, 14, 17). More recently, the emergence and worldwide spread of seasonal H1N1 viruses resistant to oseltamivir, currently the most widely used drug against influenza infections, became a considerable public health concern (15, 21, 25, 32). Monitoring the NAI resistance of influenza viruses is an ongoing public health issue since the emergence in 2009 of pandemic viruses that are resistant to M2 blockers.

Cell culture-based assays are typically not used for assessment of virus sensitivity to NAIs because of the unpredictable effect of hemagglutinin (HA) receptor binding (2, 34). Instead, drug susceptibility can be monitored by functional (biochemical) NA inhibition (NI) assays, and subsequent genotypic methods are generally required to identify the molecular marker(s) of resistance in the NA. The principle underlying the functional methods relies on the enzymatic nature of the NA, a viral surface glycoprotein and antigen. NA acts by cleaving the terminal neuraminic acid (also called sialic acid) from receptors recognized by influenza viral HA, thus facilitating the release of progeny virions from infected cells and preventing self-aggregation (29). Structurally, NAIs mimic the natural substrate, neuraminic acid, and produce tight interactions, with conserved residues of the NA active site competing with neuraminic acid for binding (11, 23). Preincubation of virus with NAIs leads to the inhibition of enzyme activity, which is detected after the addition of enzyme substrate. Most NI assays commonly used for virus surveillance utilize as substrates small synthetic conjugates that produce either a luminescent or a fluorescent signal upon cleavage by the NA enzyme. The chemiluminescent (CL) assay uses the 1,2-dioxetane derivative of neuraminic acid substrate in the influenza neuraminidase inhibitor resistance detection (NA-Star) kit (8), while the fluorescent (FL) assay employs 2′-*O*-(4-methylumbelliferyl)-*N*-acetylneuraminic acid substrate (MUNANA) (30). The results of the NI assays are expressed as the 50% inhibitory concentration ($IC_{50}$), which represents the NAI concentration that inhibits 50% of the enzyme activity of the virus. As the NA activity of clinical specimens is usually insufficient for determining the $IC_{50}$ due to a low viral content, NI assays, using either the

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substrate provided with the NA-Star kit or the MUNANA substrate, require virus propagation in cell cultures or embryonated chicken eggs. It is noteworthy that $IC_{50}$s are specific to the virus type/subtype and to the individual NAI tested (8, 19, 20, 24, 32, 37). The $IC_{50}$s obtained can be used for assessment of virus susceptibility to NAIs, including detection of resistant viruses, as well as for comparing the potencies of antiviral drugs belonging to the NAI class. Although both the CL and FL assays allow reliable detection of NAI resistance, the more recently developed CL assay was reported to be about 70 times more sensitive in detecting NA activity and has a greater linear range than the FL assay (8). The CL assay was also selected for use in the global drug susceptibility surveillance program by the Neuraminidase Inhibitor Susceptibility Network (NISN) (37, 39) and by other surveillance laboratories (28, 32). It should also be noted that $IC_{50}$s may vary even for the same virus when the NI assay is done using the NA-Star substrate (CL assay) and the MUNANA substrate (FL assay), according to reports on seasonal viruses (37). Whether one of the two assays, the CL or FL assay, more reliably predicts the level of resistance and the drug concentration required for the NA activity inhibition *in vivo* are key points of interest and remain to be elucidated.

A third assay, the colorimetric (CM) assay, which utilizes fetuin as the substrate of the NA, is typically used to determine the titer of anti-NA antibodies because small substrates do not effectively compete with antibodies (3, 31). This assay is not widely used for antiviral susceptibility testing. Unlike the NA-Star and MUNANA synthetic substrates, fetuin is a large, natural, and soluble bovine glycoprotein that contains abundant neuraminic acids at the ends of its oligosaccharide moiety (which include the presence of two residues of α2,3-linked sialic acid and one residue of α2,6-linked sialic acid) (4, 33) and has been used as a substrate in NA-catalyzed reactions (3). Given that NAIs compete with the enzyme substrate for binding to the active site, the structure of the substrate can potentially influence the outcome of the competition and, as a result, the $IC_{50}$. In this respect, fetuin may represent a better natural substrate for the enzyme-neuraminic acid attached via an α2,3 or α2,6 linkage to oligosaccharide chains on the cell surface. Furthermore, since the cleavage of each neuraminic acid is chemically converted, the CM assay can be a quantifiable method from which the resulting $IC_{50}$s would correlate more closely to the NA activity of the virus tested. Despite these apparent advantages to the use of fetuin, the CM method relies on chemical reactions that are time-consuming, cumbersome, and impractical for high-throughput use. In addition, the assay requires concentrated virus stocks for testing. Thus, fetuin is still considered an undefined substrate that does not confer sufficient sensitivity or specificity for use in routine NAI susceptibility assays (34). The potential usefulness of a large substrate such as fetuin for assessment of the NAI susceptibilities of novel H1N1 viruses or novel inhibitors remains largely unexplored.

Resistance to NAIs is not defined as clearly as that to adamantanes. In NI assays, a drug-resistant virus should have $IC_{50}$s consistently greater than the threshold value that is determined for each viral type/subtype and drug tested (27, 32, 37). Since the 2007-2008 influenza season, about a decade after the introduction of NAIs into clinical use, an NA framework mutation, H275Y (H274Y in N2 numbering), was consistently and most commonly detected in oseltamivir-resistant H1N1 viruses isolated worldwide (15, 21, 25, 32). Although the H275Y substitution represents the most-defined oseltamivir resistance marker of influenza viruses carrying the NA of the N1 subtype (35), novel NAI resistance-associated mutations—determined by elevated $IC_{50}$s in NI assays—continue to be revealed (21, 22, 32). Importantly, oseltamivir-resistant viruses from the ongoing H1N1 pandemic have been detected and reported around the world (9, 10, 26, 38). Seasonal and 2009 pandemic H1N1 viruses have the same phylogenetically distant NA gene ancestors (16), which necessitates the comprehensive assessment of the drug susceptibilities of the new pandemic viruses. Therefore, it is necessary to evaluate existing NI assays in order to better understand which assay may be the most sensitive for the detection of NAI resistance and/or the most predictive of virus susceptibility to NAIs *in vivo*.

In the present study, we assessed the susceptibilities of a panel of seasonal and pandemic H1N1 influenza viruses, including virus variants bearing the established oseltamivir resistance mutation, H275Y in the NA, against five NAIs: two FDA-approved NAIs, zanamivir and oseltamivir, and three investigational NAIs, peramivir, R-125489 (the bioactive metabolite of the prodrug CS-8958 [laninamivir]), and A-315675 (a bioactive form of the prodrug A-322278). In order to better characterize and assess the consistency of $IC_{50}$s and levels of susceptibility, these viruses were tested in the widely used CL and FL assays, as well as with the CM method.

## MATERIALS AND METHODS

**Viruses and cells.** In this study, 11 H1N1 viruses from 2009—4 seasonal (pre-pandemic) isolates (A/Washington/10/2008, A/North Carolina/02/2009, A/North Carolina/01/2009, A/Montana/02/2009) and 7 pandemic isolates (A/New York/18/2009, A/Washington/29/2009, A/Singapore/91/2009, A/Osaka/180/2009, A/Washington/29/2009, A/Hong Kong/2369/2009, A/Singapore/57/2009)—that were submitted to the World Health Organization (WHO) Collaborating Center for Surveillance, Epidemiology, and Control of Influenza at the Centers for Disease Control and Prevention (CDC), Atlanta, GA, for antigenic and antiviral susceptibility surveillance were selected as representatives for NAI susceptibility testing. Of these viruses, six viruses (two seasonal and four pandemic viruses) carried the H275Y substitution in the NA. The selection was made so that the seasonal and pandemic groups had both oseltamivir-resistant H275Y variants and their corresponding previously characterized wild-type (WT) drug-sensitive counterparts ("matching viruses"). Specifically, WT seasonal A/Washington/10/2008 and WT pandemic A/Washington/29/2009 isolates were used as the matching viruses for the respective seasonal and pandemic drug-resistant variants tested. The viruses were propagated in MDCK cells (ATCC, Manassas, VA) and were harvested at 60 h postinfection by centrifugation at 4,000 rpm for 40 min at 4°C. In order to increase the signal-to-background (S/B) ratio, the viruses were then concentrated by ultracentrifugation at 26,000 rpm for 2 h at 4°C. The pellets were subsequently suspended in 2 ml NA-Star buffer (Applied Biosystems, Foster City, CA) (see below) supplemented with 1% bovine serum albumin for use in the CM assay.

**NAIs.** Five NAIs were used in this study: two FDA-approved drugs, zanamivir (GlaxoSmithKline, Uxbridge, United Kingdom) and oseltamivir carboxylate (Hoffmann-La Roche, Basel, Switzerland), and three investigational inhibitors, peramivir (BioCryst Pharmaceuticals, Birmingham, AL), A-315675 (Abbott Laboratories, Abbott Park, IL), and R-125489 (Biota, Melbourne, Australia).

For simplicity, oseltamivir carboxylate is abbreviated to oseltamivir throughout the text. The compounds were dissolved in sterile distilled water for immediate use or were aliquoted for storage at −30°C for later use.

**NI assays.** All isolates were expanded in cell culture with limited passage (maximum of two passages). The presence or absence of the oseltamivir resistance-conferring H275Y mutation in the NA was confirmed before the viruses were tested in the NI assays.

We employed CL-, FL-, and CM-based NI assays. The formats and conditions of these assays are summarized in Table 1. The performance characteristics of

TABLE 1. Characteristics of the three NI assays used in the present study

| Assay and parameter | Result for the following assay (substrate): | | |
|---|---|---|---|
| | CL (NA-Star) | FL (MUNANA) | CM (fetuin) |
| NA activity | | | |
| NA titration | Twofold serial dilutions | Twofold serial dilutions | Twofold serial dilutions |
| Buffer | NA-Star buffer, pH 6.0 | NA-Star buffer, pH 6.0 | NA-Star buffer, pH 6.0 |
| Time and temp | 30 min, RT[b] | 30 min, 37°C | 16–18 h, 37°C |
| Virus vol (μl) | 50 | 50 | 50 |
| Substrate vol (final concn)[a] | 10 μl of NA-Star kit substrate (1.67 μM) | 50 μl of MUNANA (100 μM) | 50 μl of fetuin (5.5 mg/ml) |
| Stop solution | 60 μl of Accelerator solution (in the NA-Star kit) | 150 μl of 0.1 M glycine in 25% ethanol, pH 10.7 | Periodate oxidation with 500 μl of TBA (0.6 %) |
| Virus titer required | Low | High | High-very high (strain dependent) |
| NI assay | | | |
| Virus vol (μl) | 25 | 25 | 25 |
| Inhibitor vol (μl) | 25 | 25 | 25 |
| Signal/background ratio (linear range) | ~10-100 | In linear range (~2-20) | In linear range (~0.4-0.8 OD) |
| Inhibitor final concn (nM) | 0.03-1,000 | 0.03-1,000 | 0.03-1,000 |
| First incubation (with drug) | 30 min, 37°C | 30 min, 37°C | 1 h, 37°C |
| Second incubation (with substrate) | 30 min, RT | 30 min, 37°C | 16-18 h, 37°C |
| Assay duration | ~1 h | ~1 h | ~2 days |
| Signal (measurement) | Luminescence (0.1 s, no filter) | Fluorescent filter with excitation at 365 nm and emission at 450 nm | Photometry filter at 549 nm |

[a] The volume of the stop solution was not included in the final volume calculation.
[b] RT, room temperature.

the assays are presented in two parts: by the results of the NA enzymatic activity assay and by the results of the NI test (Table 1). The NA enzymatic activity of each individual virus was determined to establish a working virus dilution before the NI assays were conducted. In all assays, we used the NA-Star buffer system (26 mM 2-*N*-morpholinoethanesulfonic acid [MES] plus 4 mM $CaCl_2$, pH 6.0; Applied Biosystems) to dilute the virus samples, NAIs, and substrates. Ten half-log dilutions of the NAIs were freshly prepared in NA-Star buffer so that their final concentrations in the assay ranged from 0.03 to 1,000 nM. The dilutions were used immediately or were stored at 4°C for a maximum of 1 week for later use. In each assay, virus dilutions were preincubated with NAIs and subsequently incubated with the appropriate substrate.

**(i) CL assay.** The CL assay uses a 1,2-dioxetane substrate capable of reacting with the NA to release optically detectable energy as light emission, and the assay protocol has been described elsewhere (8). The assay was performed utilizing the NA-Star commercial kit, distributed by Applied Biosystems, with opaque white 96-well plates, according to the manufacturer's protocol, with minor modifications, as described previously (32). Luminescence was read for 0.1 s at a single point with a Victor 3V instrument (Perkin-Elmer, Waltham, MA). At least three independent experiments were done for each isolate.

**(ii) FL assay.** The FL assay utilizes a fluorogenic substance, MUNANA (Sigma-Aldrich, St. Louis, MO), as the NA enzyme substrate. The assay was carried out according to the method described previously (19). The released of 4-methylumbelliferone fluorescence was read from opaque black 96-well plates in the Victor 3V instrument using a fluorescence filter (Perkin-Elmer, Waltham, MA) with excitation and emission wavelengths of 365 nm and 450 nm, respectively. At least three $IC_{50}$s were determined for each isolate.

**(iii) CM assay.** The CM assay employs fetuin, a large natural glycoprotein (Sigma-Aldrich). This protein is used as an NA enzyme substrate to free sialic acid (*N*-acetylneuraminic acid [NANA]), a product from fetuin cleaved by the enzyme. In the reaction, the NANA product released from fetuin is converted to β-formol pyruvic acid by a periodate oxidation process, whose ultimate product is pigmented and can be analyzed by a spectrophotometer. The assay was initially described by Warren (36) and later modified (3). Briefly, 50 μl of 11 mg/ml fetuin solution (in NA-Star buffer) was mixed with 50 μl of 2-fold serial virus dilutions in glass tubes (7.5 mm by 10 mm), and the mixtures were incubated for 18 h at 37°C. Subsequently, 50 μl of periodate reagent (0.2 M sodium *meta*-periodate) was added, and the mixture was left for 20 min at room temperature. The oxidation process was stopped by adding 250 μl of arsenite reagent (a solution of 10% sodium arsenite, 0.5 M sodium sulfate, and 0.1 N sulfuric acid). The product of periodate oxidation (chromogen) was formed (chromophore, pink color) by boiling the mixture in a water bath with 500 μl of 6 mg/ml thiobarbituric acid (TBA) for 15 min. The chromophore was extracted with 1 ml of *n*-butanol solution containing 5% HCl (12 N) by centrifugation at 2,000 rpm for 5 min. Subsequently, 200 μl of the upper-phase extracts was transferred into clear 96-well flat-bottom plates, followed by spectrophotometric analysis at 549 nm with the Victor 3V instrument (Perkin-Elmer). A blank reaction (fetuin control, no virus) was used as a negative control.

After the NA enzymatic activity titration process, virus dilutions that gave optical density (OD) readings between 0.4 and 0.8 at 549 nm after the 18 h of incubation with fetuin were used. The NI assay was performed by mixing an equal volume of standardized virus dilution (25 μl) with serial half-log dilutions of NAIs, and the mixture was incubated at 37°C for 1 h. Fifty microliters of an 11 mg/ml fetuin solution (1:1) was added, and the mixture was shaken and incubated at 37°C for 18 h. Residual NA activity was read as described above. Each experiment was repeated at least twice.

**Pyrosequencing.** We used pyrosequencing to detect the oseltamivir resistance mutation (H275Y) in the propagated viruses used in the NI assays. The pyrosequencing reactions were performed as described previously (12, 13).

**$IC_{50}$ analysis.** Curve-fitting and $IC_{50}$ analysis for each NI assay were performed as described previously (32). Briefly, the program Robosage (a GlaxoSmithKline in-house program, kindly provided by Michael Lutz) and the following equation were used: $y = V_{max} \cdot \{1 - [x/(K + x)]\}$, where $V_{max}$ is the maximum rate of metabolism, $x$ is the inhibitor concentration, $y$ is the response being inhibited, and $K$ is the $IC_{50}$ for the inhibition curve (i.e., $y = 50\%\ V_{max}$ when $x = K$). Mean $IC_{50}$s and standard deviation (SD) values were calculated from results collected from at least three independent experiments for the CL and FL assays and two independent experiments for the CM assay.

**Statistical analysis.** The means and SDs of the $IC_{50}$ values were determined separately for each virus by drug and by assay method (see Tables 2 and 3). A one-way analysis of variance (ANOVA) was also done to compare the means of each virus by drug and by assay method (CL, FL, and CM assays) using the SAS program, version 9.2 (SAS Institute Inc., Cary, NC). Statistical significance was set at $\alpha$ equal to 0.05. *P* values are listed in Table S1 in the supplemental material.

## RESULTS

In the present study, we assessed the NAI susceptibilities of seasonal (pre-pandemic) H1N1 viruses and 2009 pandemic H1N1 viruses with or without the H275Y mutation in the NA.

The H275Y mutation is a known marker of resistance to oseltamivir in humans (15, 21, 25, 38). Both the $IC_{50}$ and the fold difference (between the $IC_{50}$s of H275Y variants and their WTs of the same origin, seasonal or 2009 pandemic isolates) were used to determine if a virus was sensitive or had altered susceptibility to NAIs. The results obtained in two NI assays utilizing small synthetic substrates, the NA-Star substrate or MUNANA, were compared to those generated using the large natural substrate fetuin. An inhibitor with a lower $IC_{50}$ than that of another antiviral in the same assay with the same virus represents a more potent NAI. A substantially elevated $IC_{50}$ in the H275Y variants compared to that in the WT viruses is indicative of possible resistance to a particular inhibitor. A consistently greater fold increase in $IC_{50}$s for the H275Y viruses tested in one NI assay compared to the $IC_{50}$s in other assays indicates a higher level of sensitivity for detecting drug-resistant viruses in that assay.

**Susceptibility to the two FDA-approved NAI drugs.** The five WT viruses demonstrated the lowest zanamivir $IC_{50}$s in the CL assay and the highest in the CM assay (Table 2). The H275Y mutants of seasonal and pandemic viruses were also susceptible to zanamivir in all three assays, and the lowest $IC_{50}$s for these viruses were seen in the CL assay (0.35 to 0.51 nM) and the highest were seen in the CM assay (1.96 to 3.61 nM). Oseltamivir $IC_{50}$s of WT viruses were the lowest in the CL assay and the highest in the CM assay (Table 2). For individual H275Y variants, however, the highest $IC_{50}$s were seen in either the FL or the CM assay. In the FL assay, seasonal and pandemic H275Y variants exerted up to 2,600-fold and ~900-fold increases in oseltamivir $IC_{50}$s, respectively, compared to those of their WT counterparts. With respect to the fold difference between the $IC_{50}$s of the WT and H275Y variants, the CL and CM assays generated results more similar to each other than to the results of the FL assay (~220- to 400-fold versus ~600- to 2,600-fold differences). With respect to the actual $IC_{50}$s, the values generated in the CL assay appeared to be consistently lower (by ~10-fold) than those generated in the FL and CM assays. Therefore, the $IC_{50}$s obtained in the FL assay were more predictive of those obtained in the CM assay (natural substrate) but overestimated the difference in the $IC_{50}$s between the WT and H275Y variants by ~2- to 8-fold.

**Susceptibility to the three investigational NA inhibitors.** Three available investigational NAIs, peramivir, R-125489, and A-315675, were tested. All viruses, including the oseltamivir-resistant H275Y variants, were highly susceptible to R-125489 in each of the three assays, as assessed by the $IC_{50}$s (Table 3). As was the case with oseltamivir, the CL assay generally detected the lowest $IC_{50}$s, followed by the FL method and, lastly, the CM assay. All seasonal and pandemic viruses both of the WT and with the H275Y mutation were highly susceptible to A-315675. Compared to their controls, the seasonal and 2009 pandemic H275Y variants exhibited marginally (~2- to 6-fold) elevated $IC_{50}$s to A-315675. All WT viruses were highly susceptible to peramivir, and a majority showed the lowest $IC_{50}$s in all three assays (Tables 2 and 3). H275Y variants of the seasonal and pandemic viruses also demonstrated reduced levels of susceptibility to peramivir in each of the assays. In the FL assay, a greater fold difference in peramivir $IC_{50}$s was seen for seasonal H275Y variants than for WT viruses (Table 3).

A statistically significant difference ($P < 0.05$) between the

TABLE 2. Susceptibilities[a] of the tested H1N1 viral neuraminidases to zanamivir and oseltamivir carboxylate

| Virus strain[b] | Subtype | NA change | Mean $IC_{50}$ (nM) ± SD[c] (fold change) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Zanamivir | | | Oseltamivir carboxylate | | |
| | | | CL assay | FL assay | CM assay | CL assay | FL assay | CM assay |
| **A/Washington/10/2008**[d] | H1N1 | WT | **0.30 ± 0.07** | **1.10 ± 0.18** | **1.64 ± 0.72** | **0.25 ± 0.04** | **0.85 ± 0.17** | **2.40 ± 0.26** |
| A/North Carolina/02/2009 | H1N1 | WT | 0.31 ± 0.15 (1) | 0.95 ± 0.06 (1) | 2.05 ± 0.75 (1) | 0.25 ± 0.05 (1) | 1.08 ± 0.31 (1) | 7.03 ± 0.29 (3) |
| A/New York/18/2009 | H1N1 pdm[e] | WT | 0.53 ± 0.05 (1) | 2.06 ± 0.39 (2) | 2.89 ± 0.68 (1) | 0.54 ± 0.05 (1) | 1.84 ± 0.32 (1) | 3.79 ± 0.33 (1) |
| **A/Washington/29/2009** | H1N1 pdm | WT | **0.45 ± 0.11** | **0.76 ± 0.13** | **2.50 ± 1.12** | **0.34 ± 0.13** | **1.59 ± 0.57** | **3.41 ± 0.10** |
| A/Singapore/91/2009 | H1N1 pdm | WT | 0.32 ± 0.04 (1) | 0.79 ± 0.13 (1) | 3.91 ± 0.72 (2) | 0.49 ± 0.04 (1) | 2.27 ± 0.30 (1) | 3.64 ± 0.10 (1) |
| A/North Carolina/01/2009 | H1N1 | H275Y | 0.42 ± 0.04 (1) | 1.16 ± 0.06 (1) | 1.96 ± 0.50 (1) | 94.40 ± 6.13 (378) | 2,050.03 ± 149.59 (2,412) | 966.27 ± 147.58 (403) |
| A/Montana/02/2009 | H1N1 | H275Y | 0.42 ± 0.05 (1) | 1.15 ± 0.23 (1) | 2.38 ± 0.50 (1) | 99.57 ± 10.14 (398) | 2,207.72 ± 210.05 (2,597) | 770.66 ± 79.65 (321) |
| A/Osaka/180/2009 | H1N1 pdm | H275Y | 0.35 ± 0.04 (1) | 1.29 ± 0.29 (2) | 3.17 ± 0.50 (1) | 93.92 ± 4.65 (276) | 1,048.21 ± 22.79 (659) | 1,333.82 ± 324.00 (391) |
| A/Washington/29/2009 | H1N1 pdm | H275Y | 0.51 ± 0.04 (1) | 0.83 ± 0.16 (1) | 2.66 ± 1.46 (1) | 95.43 ± 15.25 (281) | 1,462.51 ± 136.57 (920) | 816.52 ± 98.35 (239) |
| A/Hong Kong/2369/2009 | H1N1 pdm | H275Y | 0.43 ± 0.04 (1) | 1.02 ± 0.10 (1) | 3.61 ± 0.42 (1) | 78.12 ± 6.29 (230) | 976.71 ± 90.98 (614) | 1,204.90 ± 3.86 (353) |
| A/Singapore/57/2009 | H1N1 pdm | H275Y | 0.40 ± 0.04 (1) | 1.00 ± 0.10 (1) | 2.83 ± 0.72 (1) | 75.29 ± 3.12 (221) | 1,097.43 ± 84.31 (690) | 945.39 ± 199.17 (277) |

[a] Measured from the mean $IC_{50}$ (nM) ± SD and fold change compared to the result for the corresponding reference wild-type drug-sensitive virus.

[b] The GenBank accession numbers for the NAs of the 9 isolates are FJ686963, ACU44224, ACQ63228, ACU78206, ACU44275, GQ476129, GQ365445, ACU78206, and GQ351316. The GISAID accession numbers for the NAs of two isolates are EPI189165 and EPI189162.

[c] The $IC_{50}$s were calculated from at least three independent experiments for the CL and FL assays and at least two independent experiments for the CM assay.

[d] Boldface indicates wild-type reference drug-sensitive virus.

[e] pdm, pandemic.

TABLE 3. Susceptibilities[a] of seasonal and 2009 pandemic H1N1 viral neuraminidases to peramivir, R-125489, and A-315675

| Virus strain | Subtype | NA change | Mean $IC_{50}$ (nM) ± SD[b] (fold change) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Peramivir | | | R-125489 | | | A-315675 | | |
| | | | CL assay | FL assay | CM assay | CL assay | FL assay | CM assay | CL assay | FL assay | CM assay |
| **A/Washington/10/2008**[c] | H1N1 | WT | **0.11 ± 0.01** | **0.18 ± 0.07** | **0.75 ± 0.23** | **0.14 ± 0.01** | **0.53 ± 0.08** | **0.79 ± 0.03** | **0.16 ± 0.01** | **0.51 ± 0.15** | **1.03 ± 0.20** |
| A/North Carolina/02/2009 | H1N1 | WT | 0.08 ± 0.01 (1) | 0.19 ± 0.06 (1) | 0.70 ± 0.05 (1) | 0.17 ± 0.01 (1) | 0.61 ± 0.05 (1) | 0.86 ± 0.10 (1) | 0.18 ± 0.01 (1) | 0.51 ± 0.05 (1) | 0.75 ± 0.50 (1) |
| A/New York/18/2009 | H1N1 pdm[d] | WT | 0.12 ± 0.04 (1) | 0.32 ± 0.06 (1) | 0.41 ± 0.03 (1) | 0.49 ± 0.05 (1) | 0.96 ± 0.13 (1) | 0.74 ± 0.25 (1) | 0.43 ± 0.05 (1) | 0.74 ± 0.14 (1) | 0.90 ± 0.32 (1) |
| **A/Washington/29/2009** | H1N1 pdm | WT | **0.14 ± 0.04** | **0.20 ± 0.04** | **0.49 ± 0.10** | **0.49 ± 0.06** | **0.74 ± 0.09** | **1.57 ± 0.13** | **0.35 ± 0.05** | **0.53 ± 0.04** | **1.31 ± 0.30** |
| A/Singapore/91/2009 | H1N1 pdm | WT | 0.11 ± 0.02 (1) | 0.14 ± 0.07 (1) | 0.55 ± 0.09 (1) | 0.30 ± 0.04 (1) | 0.51 ± 0.01 (1) | 0.90 ± 0.33 (1) | 0.27 ± 0.01 (1) | 0.46 ± 0.04 (1) | 1.40 ± 0.18 (1) |
| A/North Carolina/01/2009 | H1N1 | H275Y | 13.75 ± 0.88 (125) | 149.59 ± 9.40 (831) | 105.02 ± 8.50 (140) | 0.34 ± 0.04 (2) | 0.92 ± 0.08 (2) | 0.89 ± 0.12 (1) | 0.84 ± 0.12 (5) | 1.93 ± 0.19 (4) | 2.32 ± 0.34 (2) |
| A/Montana/02/2009 | H1N1 | H275Y | 14.96 ± 0.52 (136) | 197.10 ± 24.72 (1,095) | 83.40 ± 1.34 (111) | 0.34 ± 0.02 (2) | 1.05 ± 0.01 (2) | 0.82 ± 0.11 (1) | 1.00 ± 0.34 (6) | 2.60 ± 0.08 (5) | 1.89 ± 0.31 (2) |
| A/Osaka/180/2009 | H1N1 pdm | H275Y | 13.06 ± 2.60 (93) | 97.56 ± 13.40 (488) | 194.96 ± 10.88 (398) | 0.48 ± 0.05 (1) | 1.43 ± 0.06 (2) | 3.32 ± 0.42 (2) | 0.91 ± 0.05 (3) | 2.44 ± 0.31 (5) | 4.08 ± 0.56 (3) |
| A/Washington/29/2009 | H1N1 pdm | H275Y | 12.50 ± 1.92 (89) | 150.24 ± 4.00 (751) | 162.58 ± 8.06 (332) | 0.82 ± 0.06 (2) | 1.19 ± 0.04 (2) | 2.14 ± 0.44 (1) | 1.62 ± 0.05 (5) | 1.76 ± 0.21 (3) | 2.07 ± 0.28 (2) |
| A/Hong Kong/2369/2009 | H1N1 pdm | H275Y | 9.24 ± 1.13 (66) | 128.15 ± 1.97 (641) | 161.79 ± 38.09 (330) | 0.78 ± 0.02 (2) | 1.08 ± 0.06 (1) | 2.16 ± 0.57 (1) | 1.09 ± 0.09 (3) | 1.67 ± 0.06 (3) | 3.56 ± 0.48 (3) |
| A/Singapore/57/2009 | H1N1 pdm | H275Y | 11.79 ± 0.41 (84) | 121.76 ± 2.91 (609) | 84.80 ± 14.39 (173) | 0.74 ± 0.03 (2) | 0.87 ± 0.02 (1) | 1.12 ± 0.11 (1) | 1.06 ± 0.08 (3) | 1.39 ± 0.02 (3) | 1.58 ± 0.08 (1) |

[a] Measured from the mean $IC_{50}$ (nM) ± standard deviation (SD) and fold change compared to the result for the corresponding reference wild-type drug-sensitive virus.
[b] The $IC_{50}$ values were calculated from at least three independent experiments for the CL and FL assays and at least two independent experiments for the CM assay.
[c] Boldface indicates wild-type reference drug-sensitive virus.
[d] pdm, pandemic.

mean $IC_{50}$s from the CL, FL, and CM assays was consistently observed, regardless of the virus or NAI tested (see Table S1 in the supplemental material). Therefore, the assessment of susceptibility can be influenced by the NI assay used for determination of the $IC_{50}$s.

**Potency of the three investigational NA inhibitors compared to those of the two approved drugs.** By each of the three assays, three investigational NAIs typically showed potencies comparable to or slightly higher than those of zanamivir and oseltamivir against the WT viruses (Tables 2 and 3). In contrast, the potencies of the three investigational NAIs against the H275Y variants were greater—although to various degrees—than the potency of oseltamivir, regardless of the assay used. On the basis of the CM assay data, ~80 to 190 nM peramivir was required to inhibit the enzyme activity of H275Y variants by 50%, whereas ~5- to 10-fold greater concentrations of oseltamivir were needed to produce a similar effect. The $IC_{50}$s of R-125489 and A-315675 were comparable to those of zanamivir across the three assays when both seasonal and pandemic H275Y variants were tested. These results indicate that each of the three investigational NAIs generally exhibited strong potencies in inhibiting the 2009 pandemic H1N1 viruses tested in this study. With regard to the discrimination between oseltamivir-sensitive (WT) and oseltamivir-resistant (H275Y variant) viruses, the data also indicated that the FL assay was generally more sensitive (by up to 10 times) than the CL and CM methods, which were typically equally sensitive.

## DISCUSSION

The NI assay is the primary tool used by virus surveillance laboratories to monitor susceptibility to NAIs in field isolates. In the future, the data generated using NI assays may also be used to guide antiviral treatment decisions in clinical settings. This will require established laboratory criteria and validated clear-cut thresholds for detecting clinically relevant drug-resistant viruses. In this study, we addressed several questions raised by current surveillance activities: (i) Does the choice of the NI assay have an effect on assessment of the susceptibilities of pandemic viruses to five NAIs? (ii) If $IC_{50}$s differ on the basis of the NI assay used, which one may be the most predictive of susceptibility *in vivo*? (iii) Which NI assay is the most sensitive in detecting the H275Y mutants (or, more specifically, provides a better differentiation between the WT and the H275Y variant)?

Typically, a single NI assay is used for surveillance purposes and the choice of the assay depends on many factors, such as cost, availability of equipment, virus quantity, and others. The criteria used in NAI resistance monitoring by surveillance laboratories are based on $IC_{50}$s and statistical analysis to determine outliers. On the basis of statistical analysis used for surveillance purposes, a virus is considered resistant if its $IC_{50}$ is either greater than three times the interquartile range to the right of the third quartile or greater than the mean $IC_{50}$ plus three SDs, as determined for each type/subtype and drug (27, 32). The choice of NI assay has previously been shown to affect the $IC_{50}$ of a particular virus as well as the baseline for the type/subtype. In the present study, statistically significant differences among the $IC_{50}$s determined in the CL, FL, and CM assays were seen for each virus tested. Although this does not

necessarily influence drug susceptibility surveillance because the resistance profiles are comparable in the three assays, individual viruses may appear to be more sensitive in the CL assay than in the FL or CM assay if the assessment is based on $IC_{50}$s alone. NAIs may also appear to be more potent in the CL assay than the FL and CM assays.

Among the three available NI assays, we argue that the CM assay may provide the more predictive $IC_{50}$ due to the utilization of a large natural substrate and has been considered the "gold standard" of NI assays. The CM assay is less widely used because it is cumbersome. Between the two commonly used NI assays, the CL assay is more sensitive than the FL assay in detecting the NA activity. The FL assay also requires a higher viral titer (8, 34) and may necessitate additional virus propagation prior to testing. While the NA-Star and MUNANA substrates are small, synthetic, and homogeneous, and therefore promote their use in a surveillance setting, the $IC_{50}$s determined from CL and FL methods do not necessarily correlate with the concentration of drug needed to prevent enzyme cleavage of neuraminic acid-containing receptors at the site of virus replication. Consequently, although the $IC_{50}$s and fold differences generated from the CL and FL assays can be utilized to detect resistance and/or infer reduced susceptibility, they cannot be directly correlated to the concentrations in respiratory tract secretions (or concentrations in serum) of NAIs needed *in vivo*. In this study, when WT seasonal and pandemic viruses were tested, $IC_{50}$s were typically the highest in the CM assay, regardless of the inhibitor. The CL method, which appears to offer lower $IC_{50}$s overall, can detect resistance by using the fold difference in susceptibility compared to that of sensitive viruses. However, the FL assay appears to provide the greatest difference in the $IC_{50}$s between WT and oseltamivir-resistant H275Y variants and may be the most sensitive of the three assays in detecting drug resistance. This does raise the question of whether the FL assay might overestimate the resistance of certain mutants when assessment is based on fold differences between the $IC_{50}$s of WT and mutant viruses. In this study, only viruses with dominant populations of either WT virus or viruses with the H275Y mutation were analyzed. It should be noted that the CL assay may be less suitable for detecting H275Y mutants when they are present in mixed populations with WT virus (CDC, unpublished data).

Unlike with adamantanes, several studies demonstrated that viruses resistant to one NAI may still be sensitive to other NAIs (18). Therefore, there is continued interest in the clinical development of new NAI antiviral drugs. We included in this study three investigational NAIs, peramivir, R-125489 (the bioactive form of CS-8958 [laninamivir]), and A-315675 (the bioactive form of A-322278). In each of the three NI assays, our results revealed that H275Y variants of seasonal and pandemic viruses were susceptible to zanamivir, R-125489, and A-315675 but resistant to oseltamivir and peramivir. Thus, R-125489 and A-315675 may provide alternative options for therapy against oseltamivir-resistant H1N1 viruses; however, further clinical studies are needed.

Although the CM assay is useful for the characterization of new viruses and new drugs, as presented here, and can aid in drawing potential clinical correlates, it is time-consuming and laborious, requires large quantities of concentrated viruses, and would be impractical for surveillance purposes. These caveats highlight the need to improve the methods available for detecting resistance and for accurately estimating the clinical correlation of $IC_{50}$ data determined with NI assays. Development of alternative robust and reliable functional methods for NAI susceptibility testing which would incorporate the advantages offered by the CL, FL, and CM assays but lack their limitations would be desirable, especially if they are to be used in a pandemic situation.

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We declare that we have no conflicts of interest.

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